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Immunological method for mapping genes on *Drosophila* polytene chromosomes

(biotin-labeled DNA/anti-biotin/fluorescence microscopy/immunoperoxidase localization)

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ABSTRACT A method is described for localizing DNA sequences hybridized *in situ* to *Drosophila* polytene chromosomes. This procedure utilizes a biotin-labeled analog of TTP that can be incorporated enzymatically into DNA probes by nick-translation. After hybridization *in situ*, the biotin molecules in the probe serve as antigens which bind affinity-purified rabbit antibiotin antibodies. The site of hybridization is then detected either fluorimetrically, by using fluorescein-labeled goat anti-rabbit IgG, or cytochemically, by using an anti-rabbit IgG antibody conjugated to horseradish peroxidase. When combined with Giemsa staining, the immunoperoxidase detection method provides a permanent record that is suitable for detailed cytogenetic analysis. This immunological approach offers four advantages over conventional autoradiographic procedures for detecting *in situ* hybrids: (*i*) the time required to determine the site of hybridization is decreased markedly; (*ii*) biotin-labeled probes are chemically stable and give reproducible results for many months; (*iii*) biotin-labeled probes appear to produce less background noise than do radiolabeled probes; and (*iv*) the resolving power is equal to and often greater than that achieved autoradiographically.

In situ hybridization, initially developed by Gall and Pardue (1) and John *et al.* (2), has proven to be a valuable method for determining the cellular or chromosomal location of hybridized nucleic acids (3–10). Standard *in situ* hybridization protocols use radiolabeled RNA or DNA probes and autoradiographic methods of detection or quantification. By using probes of high specific activity under conditions such that hybridization "networks" are formed (8–12), it is now possible to localize unique sequences in mammalian chromosome spreads after autoradiographic exposures of 5–22 days (9, 10). However, the inherent drawbacks of radiolabeled probes—notably chemical lability due to radiolytic decomposition, concern for personnel safety, and disposal problems—make it desirable to have sensitive methods for detecting polynucleotide sequences that do not rely on the use of radioisotopes, especially for routine applications in clinical medicine.

Several groups have attempted to develop such procedures. Cheung *et al.* (13) generated a fluorescent signal by coupling latex microspheres containing both poly(U) and dansylated fluorochromes to a polyadenylylated mRNA probe. Fluorescent signals were also produced by Rudkin and Stollar (14) by using antibodies against DNA·RNA hybrids in conjunction with an immunofluorescent antibody sandwich and by Bauman *et al.* (15–17) who used RNAs that were labeled at the 3′ end with fluorescein or rhodamine. Davidson and associates (18–20) chemically crosslinked biotin to RNA with cytochrome *c* or polyamine bridges and used these RNA–biotin complexes as hybridization probes. The sites of hybridization were visualized in the electron microscope through the binding of avidin-ferritin or avidin-methacrylate spheres. Wu and Davidson (21) recently described an additional method for gene mapping on *Drosophila* polytene chromosomes by using the electron microscope. Colloidal gold spheres were coated with protein and poly(dT)-tailed heterologous DNA and used to identify the hybridization sites of poly(dA)-tailed *Drosophila* DNA probes. Although each of these approaches was at least partially successful, a simpler and more general method for detecting nonradioactively labeled DNA or RNA probes would be desirable.

The specificity and tenacity of the biotin–avidin interaction (22) makes biotin an attractive candidate as an affinity reagent for tagging nucleic acids. We recently reported the synthesis of dUTP and UTP analogs that contain a biotin molecule covalently attached to the C-5 position of the pyrimidine ring through an allylamine linker arm and demonstrated that these nucleotides can function as efficient substrates for various DNA or RNA polymerases *in vitro* (23). In addition, biotin-substituted polynucleotides were shown to have denaturation and reassociation characteristics that were compatible with their use as hybridization probes (23).

In this report, we describe the first stages in the development of a generalized method for *in situ* hybridization based on biotinized polynucleotides as specifically applied to *Drosophila* polytene chromosomes. A preliminary account of this work was presented elsewhere (24).

MATERIALS AND METHODS

Standard nucleoside 5′-triphosphates were obtained from P-L Biochemicals. Radiolabeled compounds were products of New England Nuclear or Amersham. *Escherichia coli* DNA polymerase I was purchased from Boehringer Mannheim. Egg white avidin, biotin, ovalbumin, diaminobenzidine, and Hanker–Yates reagent were obtained from Sigma. Fluorescein-labeled goat anti-rabbit IgG (FITC-GαRIgG) and goat anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Miles-Yeda. Peroxidase-conjugated rabbit anti-goat IgG was a product of Polysciences (Warrington, PA). Peroxidase-conjugated sheep anti-rabbit IgG was the gift of P. Nakane.

Five of the cloned DNAs used in these studies (λDm 117, λDm 221, λDm 60, λDm 104, and λDm 89) encode genes that are specifically expressed in the fat bodies of third-instar larvae (25). They were obtained from a collection of randomly sheared *Drosophila melanogaster* genomic DNA fragments inserted into the Charon 4 phage vector (26). The *Drosophila* DNA in-

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Abbreviations: P_i/NaCl, phosphate-buffered saline; NaCl/Cit, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate; FITC-GαRIgG, fluorescein isothiocyanate-labeled goat anti-rabbit IgG; kb, kilobase(s).

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serts in these clones vary in size from 16 to 19 kilobases (kb).

Clones pPW 539 and p14C4 (provided by Otto Schmidt) contain specific tRNA genes cloned into pMB9 and pSF 2124, respectively. Clone pAC 104 (provided by V. Pirotta) encodes a transposable element cloned into the plasmid pACYC 184.

Preparation of Hybridization Probes. *In vitro* nick-translation reactions were done essentially as described by Rigby *et al.* (27) with certain modifications. DNA probes (0.1–2.0 μg per reaction) were incubated in a 50-μl reaction mixture containing 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, bovine serum albumin at 50 μg/ml, 10 mM 2-mercaptoethanol, and 25 μM dATP, dGTP, dCTP, and either TTP or biotin-dUTP. Pancreatic DNase I (Sigma, type I) was added to a final concentration of 0.01 μg/ml. *E. coli* DNA polymerase I (New England BioLabs) was added immediately (11 units in 50-μl reaction) and the mixture was incubated at 14°C. In kinetic experiments, aliquots were removed at the indicated times and trichloroacetic-acid precipitates were assayed for radioactivity in scintillation fluid. Otherwise, the reactions were terminated after 90 min by addition of EDTA to a final concentration of 20 mM and incubation at 55–60°C for 10 min. Phenol extraction was avoided because heavily substituted polymers are extracted into the phenol layer, and even lightly substituted polymers may remain at the phenol/H_2O interface. Unincorporated nucleotide was removed either by gel filtration over Sephadex G-50 equilibrated in 10 mM Tris (pH 7.5) or by successive ethanol precipitations. Probes prepared by this protocol routinely contained between 8% and 40% of their thymidine residues substituted with the biotin-labeled analog.

Purification of Rabbit Anti-Biotin Antibody. Biotin–bovine serum albumin complex, prepared as described (28), was used to immunize rabbits by a modification of the Berger procedure (29). Biotin–albumin, at 2 mg/ml in 0.9% NaCl, was combined with an equal volume of Freund's complete adjuvant (Difco, no. 0638-60) and the mixture was emulsified by repeated passage through an 18-gauge needle. Initial immunizations were with 1.2 ml of emulsion (0.1 ml intramuscularly into the lower and upper parts of each limb and 0.1 ml intradermally three times in a vertical row on either side of the midline). The animals were given booster injections every 14 days with 1.2 ml of biotin–albumin (2 mg/ml in 0.9% NaCl) as above. After 6 weeks the rabbits were producing antibiotin antibodies as determined by Ouchterlony immunodiffusion, and 30–40 ml of blood was collected by cardiac puncture 10 days after each booster injection. The blood was allowed to clot for 1 hr at 37°C and then was refrigerated for 2–24 hr. Clotted material was removed by centrifugation and serum was stored at −20°C.

Purification of rabbit antibiotin was done by affinity chromatography on columns of ovalbumin-Sepharose and biotin-ovalbumin-Sepharose. Cyanogen bromide-activated Sepharose 4B (30) was incubated with an equal volume of protein solution (2 mg/ml) in 0.1 M $NaHCO_3$ for about 18 hr at 4°C. After coupling, unreacted sites were blocked by incubating the resin in 0.1 M glycine in phosphate-buffered saline (P_i/NaCl) for 2 hr at room temperature. The resin was washed successively with P_i/NaCl, 3 M KSCN in P_i/NaCl, 0.2 M acetic acid, and 50 mM NaOH and then equilibrated with P_i/NaCl. Approximately 10 ml of immune serum was loaded onto a 5-ml bed of ovalbumin-Sepharose t remove any ovalbumin binding component. The flow-through fraction from this column was applied directly to a 5-ml bed of biotin-ovalbumin-Sepharose and the resin was washed extensively with P_i/NaCl until the flow-through contained no detectable protein. Protein bound to the resin was then eluted with 3 M KSCN in P_i/NaCl, concentrated in an equilibrated in P_i/NaCl. The antibody recovered in the void volume was adjusted to approximately 0.5 mg/ml in P_i/NaCl. Bovine serum albumin (fraction V, Sigma) was added to a final concentration of 5 or 10 mg/ml and the solution was stored at −20°C. The purified antibody preparation was at least 70% IgG as determined by chromatography on IgG-specific DEAE-Affi-Gel blue (Bio-Rad). In Ouchterlony immunodiffusion assays, precipitin lines were seen only upon reaction of purified antibody with biotin-labeled albumin or DNA-nick translated with biotin-dUTP. Similar specificity for biotin-DNA was observed in immunoprecipitation assays with formalin-fixed *Staphylococcus aureus* (23).

Preparation of Chromosome Spreads. *Drosophila* polytene chromosome spreads were prepared essentially as described by Pardue and Gall (31) from salivary glands derived from third-instar larvae of either the giant strain (gtw^c/gt^{hl}), Oregon R, or Canton S. The acetylation step described by Hayashi *et al.* (32) to decrease nonspecific binding of nucleic acids was included just before the chromosomes were denatured in alkali.

Hybridization Conditions. Biotin-labeled probe DNA was hybridized to polytene chromosome spreads by using one of the following three protocols.

Method 1. DNA was dissolved at a final concentration of 0.4–2 μg/ml in 20 mM Tris·HCl, pH 6.8/0.3 M NaCl containing 100–250 μg of heterologous carrier DNA (herring sperm) per ml. The DNA was denatured by boiling for 5 min and then quick-cooled on ice. Approximately 25 μl of this mixture (containing 10–60 ng of probe) was used per slide under an acid-washed 22-mm^2 coverslip. Hybridization was done in a sealed moist chamber at 65°C for 10–12 hr. Nonspecifically bound material was removed after hybridization by washing three times in 2× standard saline citrate (NaCl/Cit) at 60°C for 10 min each, followed by two washes with 2× NaCl/Cit for 15 min each at room temperature and three washes in P_i/NaCl for 5 min each.

Method 2. The labeled DNA was dissolved at a final concentration of 4 μg/ml in 0.3 M NaCl/5 mM $MgCl_2$/50 mM NaP_i, pH 7.0/Denhardt's solution (33) containing 200 μg of sonicated salmon sperm DNA per ml. The DNA was denatured and hybridized as in method 1. Nonspecifically bound DNA was removed by washing three times in 2× NaCl/Cit at 60°C for 20 min each, followed by three washes with P_i/NaCl for 5 min each at room temperature.

Method 3. Probe DNA was dissolved at a final concentration of 2 μg/ml in a solution 40% (vol/vol) formamide in 4× NaCl/Cit containing 200 μg of sonicated salmon sperm DNA per ml 10% (wt/vol) dextran sulfate. The DNA was denatured by boiling for 2 min and hybridized at 42°C for 4–6 hr. Nonspecifically bound probe was removed as described in method 2.

Detection of Hybridized Probe. In all cases, the *Drosophila* chromosome spreads were incubated with 25–50 μl of rabbit antibiotin (2.5 μg/ml in P_i/NaCl containing 10 mg of bovine serum albumin per ml) at 37°C for 2–4 hr followed by two rinses with P_i/NaCl for 5 min each.

For direct fluorescence detection the slides were incubated with 25–50 μl of FITC-GαRIgG diluted 1:100 in P_i/NaCl/albumin at 37°C. After rinsing in P_i/NaCl, the slides were counterstained with 0.005% Evan's blue (Allied Chemical) in P_i/NaCl containing 1% fetal calf serum for 2 min, rinsed in P_i/NaCl, and mounted in 1 M Tris, pH 8.0/glycerol, 1:9 (vol/vol). The slides were then examined by phase-contrast and fluorescence microscopy (Nikon Optiphot epilluminated fluorescence microscope). Photography was with Kodak Ektachrome 400 film.

Histochemical detection was done by one of the following

37°C for 1 hr. After rinsing in P_i/NaCl, the slides were stained with 3,3'-diaminobenzidine (0.1 mg/ml 50 mM Tris·HCl, pH 7.5)/0.03% H_2O_2 at room temperature for 5–30 min. The slides were rinsed with P_i/NaCl and stained with Giemsa as described by Pardue and Gall (31). Alternatively, after incubation with FITC-GαRIgG, the slides were incubated with peroxidase-conjugated rabbit anti-goat IgG (diluted 1:100 in P_i/NaCl/albumin) at 37°C for 1–2 hr. The slides were rinsed in P_i/NaCl and stained with 0.05% Hanker–Yates reagent (34)/0.01% H_2O_2/10 mM Tris·HCl, pH 7.6, at room temperature for up to 45 min. The slides were rinsed, stained as above, and photographed with Kodak Tri-X Pan film in a Zeiss light microscope.

RESULTS

D. melanogaster polytene chromosomes were chosen as the test system for establishing whether probes containing biotinized nucleotides could be detected after *in situ* hybridization, by biotin-specific immunological or affinity reagents. Several cloned *Drosophila* sequences were tested, of which most have already been assigned to specific bands on the *Drosophila* chromosome map by conventional *in situ* hybridization protocols employing autoradiographic detection (see legends to Figs. 1 and 2).

In all cases, the intact clones, including both vector and inserted *Drosophila* sequences, were nick-translated in the presence of biotin-dUTP. Occasionally, [^{3}H]dCTP was included in the nick-translation reaction mixture to provide both autoradiographic and immunological localization on a single chromosome spread.

It was our original intention to use avidin conjugated to various indicator molecules in order to localize biotin-labeled hybridization probes. However, our initial negative results with standard avidin preparations, reinforced by reports that avidin binds nonspecifically to DNA and chromatin (35, 36), led us to develop an alternative detection system using affinity-purified antibiotin antibodies. Only affinity-purified antibodies were used in these studies because whole serum exhibited higher levels of nonspecific background binding.

Fig. 1 establishes the clarity, specificity, and resolution of the hybridization signals obtained by indirect immunofluorescence. Signals of this quality are usually obtained with at least 90% of the chromosome spreads on a single slide. The time of antibody incubations was varied from 0.5 to 18 hr for the first

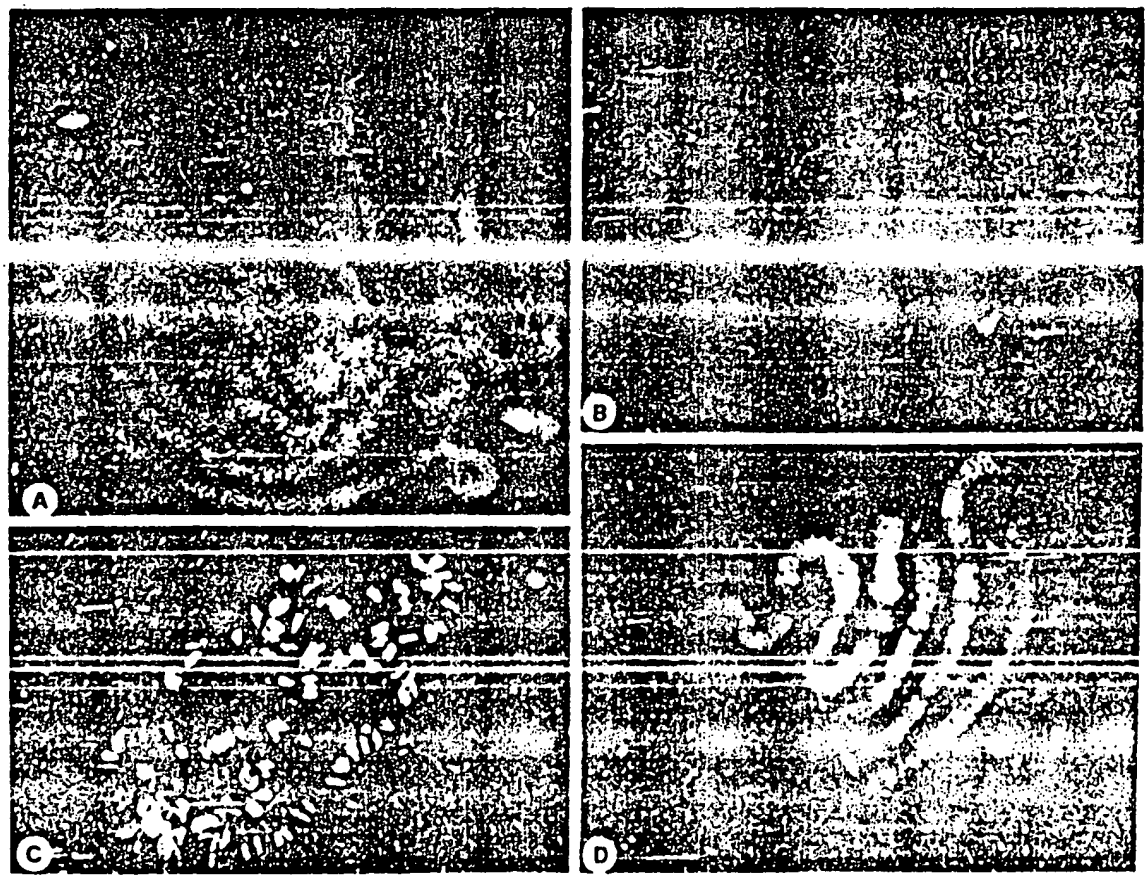

FIG. 1. Detection of cloned *Drosophila* DNA sequences by indirect immunofluorescence. Biotin-labeled DNA probes were hybridized to polytene chromosomes of the Canton S strain according to method 1. (*A*) Hybridization signal obtained with clone pPW539, which contains a 22-kb fragment encoding a methionine tRNA gene known to map to band 61 D on chromosome 3L (37). (*B*) Fluorescent signal obtained after hybridization with clone p14C4, which contains an 18-kb fragment encoding arginine and asparagine tRNAs known to map to band 84 F on chromosome 3R (38). (*C*) Numerous loci throughout the *Drosophila* genome that hybridize with the 8.9-kb fragment of the transposition element B104 present in clone pAC104 (39). (*D*) Hybridization signal obtained with a pBR322 clone, containing 3.0 kb of *Drosophila* DNA that was randomly selected from a genomic library. The probe hybridized to three loci, the two minor signals having only 10–20% of the intensity of the major site as determined by densitometry. Fluorescent micrographs were taken after counterstaining with Evans blue.

antibody and from 0.5 to 4 hr for the second antibody. The minimum incubation times for a strong fluorescent signal appeared to be 2 hr for the first antibody and 1 hr for the second antibody. Shorter incubation times gave signals that could be seen directly in the microscope but sometimes were difficult to photograph. The Evans blue counterstain, which outlines the general chromosome structure in red, was clearly distinguishable from the yellow-green fluorescence which marks the hybridization sites. Thus, the general chromosome fluorescence shown in Fig. 1 does not reflect nonspecific binding of the FITC-labeled antibody to chromosomal material.

The sequential addition of all three reagents (biotin-DNA, rabbit antibiotin antibody, and FITC-GαRIgG) was essential to generate a fluorescent signal. No bands were observed after hybridization with biotin-DNA if the primary incubation with antibiotin antibody was omitted or if it was replaced by an incubation with nonimmune rabbit serum. Similarly, no signal was observed after hybridization of nonbiotinized probes, with either antibiotin or control antibodies.

Although the immunofluorescent detection protocol revealed hybridization loci with speed and precision, it does have several drawbacks. The fluorescent signal can bleach within minutes when exposed to a high-intensity light source, and it slowly fades on storage, even in the dark. Furthermore, mapping a gene locus requires careful comparison of separate phase-contrast or conventional light micrographs with the corresponding fluorescent micrographs, because Giemsa staining of chromosomes abolishes the fluorescent signal. In order to provide a stable hybridization signal that would be compatible with high-resolution cytogenetic analysis, we used secondary antibody-peroxidase conjugates to mark the hybridization locus. The catalytic activity of the peroxidase enzyme converts diaminobenzidine and other substrates, such as Hanker–Yates reagent, into highly insoluble brown precipitates which can be clearly distinguished from the blue cytologic bands revealed by Giemsa staining.

Fig. 2 shows several examples in which an immunoperoxidase detection protocol was used for hybrid localization. The mapping of the P1 gene to the 70 D region of chromosome 3 (Fig. 2*D*) illustrates the clarity and specificity that can be achieved routinely. In addition, sequences that are not localized in sharply defined chromosome bands—e.g., at chromosome telomeres (Fig. 2 *A* and *B*) or across chromosome constrictions (Fig. 2*F*)—can be detected with high efficiency. One additional attribute of the immunoperoxidase detection method is that the kinetics of enzyme product deposition at the hybridization site can be monitored by light microscopy. This permits one to control the intensity of the "exposure" so that an optimal signal-to-noise ratio can be achieved. However, the generation of excessively large precipitates should be avoided because it can result in the artefactual appearance of doublet or triplet bands of apparent hybridization. For example, Fig. 2*E* shows the precipitates generated at the LSP-2 and P1 loci after cohybridization with a 20:1 mixture of biotin-labeled LSP-2 and P1 DNA, respectively, under conditions such that hybridization at the P1 locus is nonsaturated. The single LSP-2 gene locus (solid arrowhead) appears to be very broad; when examined at high resolution, it can be resolved as a triplet band.

The majority of the hybridization results presented here were obtained with DNA clones that contained between 8 and 22 kb of *Drosophila* DNA. However, both the immunofluorescence and immunoperoxidase detection protocols have been used successfully for mapping DNA clones containing as few as 200 base pairs of *Drosophila* sequence (unpublished results). Thus, the method has a sensitivity comparable to that which can be obtained by using probes of high specific radioactivity. By using a hybridization cocktail that contains formamide and dextran sulfate (method 3), reagents known to increase the efficiency and rate of hybridization (9, 11, 12), in conjunction with either of the immunological detection protocols described here, it is now possible to map most *Drosophila* DNA sequences within 8–9 hr.

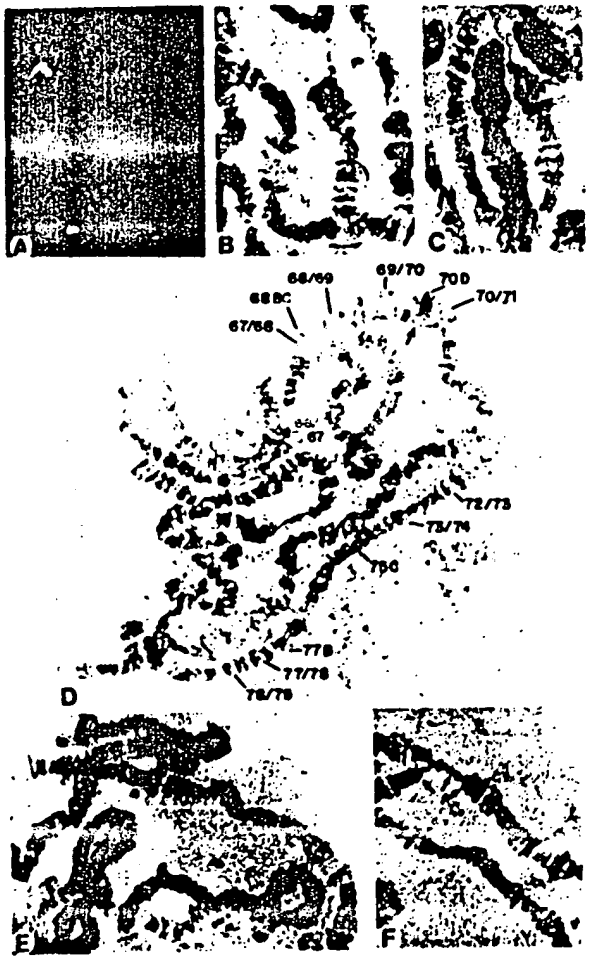

FIG. 2. Detection of hybridized DNA probes by indirect immunofluorescence (*A*) and by immunoperoxidase reactions (*B–F*). (*A*) Clone $\lambda_{DM}60$, which contains the LSP-1γ gene, was hybridized to giant strain polytene chromosomes according to method 2. Detection was by indirect immunofluorescence without Evans blue counterstaining. (*B–F*) The sites of hybridization were detected with Hanker–Yates reagent after incubation with FITC-GαRIgG and peroxidase-conjugated rabbit anti-goat IgG. Chromosomes were stained with Giemsa before photography. (*B*) Same chromosome as in *A* but after the addition of the peroxidase-antibody conjugate and the development of the indicator precipitate. (*C*) Biotin-labeled $\lambda_{DM}89$ DNA, which contains the P-6 gene on an 18-kb insert, hybridized according to method 2. The P-6 gene maps to the 30B region of chromosome 2. (*D*) Biotin-labeled $\lambda_{DM}117$ DNA, which contains the P1 gene, hybridized to polytene chromosomes of the Oregon R strain according to method 3. The P1 gene maps to the 70D region on chromosome 3. (*E*) Signal generated after cohybridization of a 20:1 mixture of $\lambda_{DM}104$ DNA (containing the LSP-2 gene) and $\lambda_{DM}117$ DNA (containing the P1 gene) to giant strain polytene chromosomes according to method 2. The LSP-2 gene maps to the 68E region on chromosome 3. (*F*) Hybridization of biotin-labeled $\lambda_{DM}221$ DNA, containing the LSP-1α gene, to giant strain polytene chromosomes according to method 2. The LSP-1α gene maps to the 11A region on the X chromosome.

DISCUSSION

We have described a method for the *in situ* localization of specific sequences on chromosomes which exploits the interaction between biotinyl nucleotides and antibiotin antibodies. This technique offers four advantages over conventional autoradiographic detection procedures, particularly as applied to *Drosophila* polytene chromosomes: marked decrease in the time required for localization, improved resolution, less nonspecific background, and chemically stable hybridization probes that give reproducible results over a period of many months. Although the procedure as described has used only nick-translated DNA probes, it is also possible to use biotin-labeled cRNA probes prepared by transcribing single-stranded DNA sequences inserted into cloning vehicles such as M13. Furthermore, because a large number of biotinyl nucleotides can be introduced into DNA or RNA polymers without significantly altering their hybridization characteristics (23), standard hybridization protocols need be modified little, if at all, when biotinized probes are used.

Further exploitation of the interaction between nucleotide analogs and antinucleotide antibodies should make it possible to do high-resolution mapping on the *Drosophila* chromosome. For example, if two independent probes that map close together on the chromosome are labeled with different "haptene"-substituted nucleotide analogs, cohybridized to polytene spreads, and detected by antihaptene antibodies (tagged directly or indirectly with different fluorochromes or enzyme indicators), each hybridization locus can be identified by a different color. A rapid method for discriminating between cohybridized probes should be of significant utility, especially for characterizing cloned sequences obtained during chromosome "walks" from one locus toward another.

The immunohistochemical detection methods also have been used successfully for localizing specific DNA sequences in mammalian metaphase chromosomes by both light and electron microscopy (unpublished data), for detecting cRNA probes hybridized to transcriptionally active regions on lampbrush chromosomes of the newt *Notophthalamus* (J. Gall, personal communication), and for detecting specific RNA and DNA sequences in virus-infected cell cultures and tissue sections (unpublished data). These results suggest that the combination of biotin-labeled polynucleotide probes and antibiotin antibody detector reagents will have general utility in the localization and quantification of nucleic acid sequences *in situ*. However, the methods used for labeling the probe and for detecting the probe after hybridization are still prototypes and can be improved. For example, we have observed that probes containing pyrimidine nucleotides that have a longer linker arm between the biotin moiety and the pyrimidine ring interact with antibiotin antibodies or avidin with greater affinity than do the biotin-labeled DNA probes used in this study (unpublished data). In addition, we have recently obtained avidin derivatives or avidin analogs—e.g., streptavidin from *Streptomyces avidinii* (40)—which do not exhibit nonspecific binding to DNA or chromatin.

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